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Toxic threshold of dietary microcystin (-LR) for quart medaka

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ABSTRACT

This study was designed to estimate the toxic threshold of male and female fish to microcystins based on different biomarkers. Japanese medaka (*Oryzias latipes*) were fed dietary Microcystin-LR (0, 0.46, 0.85, 2.01 and 3.93 μg MC-LR/g dry diet for 8 weeks at 25 °C. The results revealed that dietary MC-LR inhibited growth at the end of 8 weeks. The survival of embryos and the RNA/DNA ratio of whole fish decreased significantly ($P < 0.05$) in fish fed 3.93 μg MC-LR/g dry diet. Heat shock protein (Hsp60) expression was induced in the liver of female and male fish fed diets containing ≥0.85 and 0.46 μg MC-LR/g diet, respectively. The activity of liver caspase 3/7 was significantly higher in female fish fed 3.93 μg MC-LR/g diet and in males fed 2.01 MC-LR μg/g dry diet than fish fed the control diet. The threshold for inhibition of liver protein phosphatase expression was lower in female (2.01 μg/g diet) than that in male fish (3.93 μg/g diet). Histopathological examination showed significant single-cell necrosis in female and male medaka fed diets containing 0.85 and 3.93 μg MC-LR/g diet, respectively. Based on different biomarkers, this study demonstrated that dietary MC-LR is toxic to Medaka and the effects are gender dependent.

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1. Introduction

Cyanobacteria (*Microcystis aeruginosa*) blooms are known to cause deleterious effects in aquatic ecosystems including zooplankton, fish, waterfowl, mammals, and humans. The most common and well-studied cyanotoxin is the hepatotoxin microcystin-LR (MC-LR), which has been on the rise in abundance and distribution in the upstream portion of the San Francisco Estuary since 1999 (Lehman et al., 2005). Of the 70+ isoforms of microcystins identified, MC-LR is the most studied, toxic and common (Zurawell et al., 2005). Although MC-LR specifically targets liver (Carmichael, 1995), it also impairs the function of other organs such as kidney, gills and the gastrointestinal tract (Rabergh et al., 1991; Kotak et al., 1996; Carbis et al., 1997). Several studies have reported the impact of MC-LR on the reproductive system in mice (Ding et al., 2006), rat (Li et al., 2008; Xiong et al., 2009), and fish (Baganz et al., 1998). Furthermore, field investigations on aquatic invertebrates and fish have strongly implicated the adverse effect of MC-LR on reproductive organs (Chen and Xie, 2005; Zhang et al., 2009).

MC-LR toxicity is the result of inhibition of phosphatase (PP1/PP2A) activity (Runnegar et al., 1993) and destruction of the cytoskeleton, which ultimately leads to cytotoxicity, interruption of cell division, and tumor-promoting activity (Carmichael, 1994; Humpage and Falconer, 1999; Fischer et al., 2000). Microcystin toxicity is also due to oxidative stress that causes apoptosis or necrosis depending on exposure concentration and duration (Ding and Ong, 2003; Li et al., 2005, 2007; Morena et al., 2005; Cazenave et al., 2006).

Most investigations on microcystin toxicity were based on aqueous (Tencalla et al., 1994), one time force-feeding (Tencalla and Dietrich, 1997), short term dietary exposure bioassays (Juhel et al., 2006) that determine the acute effects of microcystins on fish (Sun et al., 2008). Only

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limited information is available on the chronic dietary effect of microcystin on fish (Xie et al., 2004; Zhao et al., 2006) which was shown to be the major route of microcystin toxicity for fish in the natural environment (Zhang et al., 2009). In addition, there has been growing evidence to indicate that a suite of environmental chemicals, both anthropogenic and those occurring naturally, have the potential to alter endocrine-mediated sexual development resulting in disruption of gonadal sex differentiation and gametogenesis (Shutt, 1976; Bergeron et al., 1994; White et al., 1994; Kelce and Wilson, 1997; Gray et al., 2006). Medaka (*Oryzias latipes*) is a well-studied, highly-responsive fish model that has been used successfully to characterize acute and chronic toxicity in fish. Recent studies have also demonstrated that medaka is an appropriate model for studying toxic effects of cyanobacteria (Jacquet et al., 2004; Huynh-Delerme et al., 2005; Escoffiera et al., 2007; Mezhoud et al., 2008). To our knowledge, there is no information on gender effects of MC-LR on fish. The purpose of this study was to determine the dietary toxic threshold of MC-LR on male and female medaka based on integrated biomarkers. We hypothesized that 1) MC-LR affects reproduction performance in fish and 2) the sensitivity to the toxic effect of MC-LR is gender dependent.

2. Materials and methods

2.1. Experimental diets

Five test diets were prepared to contain graded levels of microcystin-LR (MC-LR), Dietary levels were: 0, 0.46, 0.85, 2.01 and 3.93 μg/g dry diet, respectively, and the levels of MC-LR were analyzed based on the method described by Hu et al. (2008). MC-LR (*M. aeruginosa*, $C_{49}H_{74}N_{10}O_{12}$) was purchased from EMD Biosciences Inc. (San Diego, CA, USA). The control diet was formulated without supplementation of MC-LR. The basal diet contained (g/kg): vitamin free casein, 310; wheat gluten, 150; dextrin, 272; egg albumin, 40; soy lecithin, 52; non nutritive bulk, 36; cod liver oil, 50; corn oil, 20; vitamin premix, 40; and mineral premix, 30. Except for the vitamin and mineral premixes, which were purchased from ICN (Biomedical, Inc., Irvine, CA), all other ingredients were obtained from U.S. Biochemical Corporation (Cleveland, OH, USA). The dry ingredients were thoroughly mixed before the oil was added. Double distilled water containing different concentrations of MC-LR (previously dissolved in methanol) was added to make wet dough. Pellets were prepared, freeze-dried and stored in the dark at −20 °C until use (Deng et al., 2008).

2.2. Dietary exposure of MC-LR

Embryos of Japanese medaka (*O. latipes*) were collected from our medaka culture system and separated by gender within 4 days post fertilization based on sex-linked coloration (Wada et al., 1998). After hatching (usually 8–10 days post-fertilization), larvae were cultured in a recirculation system with 20 fiberglass tanks (20 L per tank) and fed three times daily with the basal purified diet until used for the exposure study. Water flow-rate and temperature was 0.9 L/min and 25 ± 1 °C, respectively. Water quality including dissolved oxygen (8.3 mg L^{-1}), pH (7.8), water hardness (120 mg L^{-1}), and ammonium (not detectable) were monitored weekly.

The dietary exposure of MC-LR was conducted using 7-week old medaka. The initial body weight of fish was 82 ± 2 mg. Four tanks were randomly assigned to each dietary treatment with 2 tanks per gender and 100 fish per tank. Fish were fed twice per day (0900 and 1500 h) based on 5% of body weight daily. Water flow was stopped during feeding to prevent contamination of the recirculation system with dissolved MC-LR. The waste, uneaten feed and 50% of the water were siphoned from each tank 30 min after each feeding. To ensure that dissolved MC-LR from the diets did not contribute to any significant health effect to the fish, charcoal filters were changed weekly and 100% of the water in the recirculation system was replaced each day. Care, maintenance, handling, and tissue sampling of the fish followed the protocols approved by the University of California-Davis Animal Care and Use Committee.

2.3. Growth and reproduction

Fish were weighed at the end of 2, 4 and 8 weeks of feeding to estimate fish growth. In addition, at the end of 4 weeks of feeding, 30 females and 20 males fed the same dietary MC-LR concentration were mixed and allowed to breed to estimate reproductive performance. The fecundity (egg production per female) and survival of embryos were monitored each morning.

2.4. Sampling

At the end of 8 weeks, fish were killed by an overdose of MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA). Fish were weighed and measured for length, then separated into three groups. Group 1): 5 females and 5 males from each replicate tank were fixed in 10% neutral buffered formalin for histopathological examination by the method described by Teh et al. (2004). Group 2): 5 females and 5 males from each replicate tank were dissected to remove liver and ovary tissues. Liver and ovary tissues were weighed to estimate liver and ovarian somatic indices. Tissues were frozen in liquid nitrogen and stored at −80 °C until used for stress protein, protein phosphatase analysis and enzyme assay. Group 3): 5 females and 5 males from each replicate tank were frozen in liquid nitrogen and stored at −80 °C until pulverized with liquid nitrogen using a Freezer/Mill (SPEX SamplePrep, L.L.C., Metuchen, NJ, USA) and used for RNA/DNA analysis to estimate fish growth and recent feeding status.

2.5. Sample analysis

Fixed samples for histopathology were dehydrated in a graded ethanol series and embedded with both surgically cut sections face down in paraffin. Serial longitudinal sections (3 μm) were stained with hematoxylin and eosin (H&E), and lateral views of liver, kidney and gonads were screened for a variety of histopathological features and lesions. Livers were analyzed for lesions of glycogen depletion (GD), lipidosis (LIP), and single-cell necrosis